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Original Articles

Hypoxia enhances migration and invasion in glioblastoma by promoting a mesenchymal shift mediated by the HIF1α–ZEB1 axis

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ABSTRACT

Glioblastoma (GBM) is the most common brain tumor in adults and the mesenchymal GBM subtype was reported to be the most malignant, presenting severe hypoxia and necrosis. Here, we investigated the possible role of a hypoxic microenvironment for inducing a mesenchymal and invasive phenotype. The exposure of non-mesenchymal SNB75 and U87 cells to hypoxia induced a strong change in cell morphology that was accompanied by enhanced invasive capacity and the acquisition of mesenchymal marker expression. Further analyses showed the induction of HIF1α and HIF2α by hypoxia and exposure to digoxin, a cardiac glycoside known to inhibit HIF1/2 expression, was able to prevent hypoxia-induced mesenchymal transition. ShRNA-mediated knockdown of HIF1α, and not HIF2α, prevented this transition, as well as the knockdown of the EMT transcription factor ZEB1. We provide further evidence for a hypoxia-induced mesenchymal shift in GBM primary material by showing co-localization of GLUT1, ZEB1 and the mesenchymal marker YKL40 in hypoxic regions of the tumor. Collectively, our results identify a HIF1α–ZEB1 signaling axis that promotes hypoxia induced mesenchymal shift and invasion in GBM in a cell line dependent fashion.

Introduction

Glioblastoma (GBM) is the most aggressive brain tumor, and despite multimodal treatment with surgery, radiation and chemotherapy patients generally show incurable relapse of the disease [1]. The median survival time of patients with GBM is <16 months even after optimal treatment [2]. Recent advancements in genomic sequencing and transcriptome analysis have stratified GBM into different molecular subtypes [3,4], of which the mesenchymal (MES) and proneural (PN) subtypes appear to be the most pronounced [5]. A mesenchymal phenotype in GBM has been associated with tumor aggressiveness and elevated invasive potential [4,6]. Interestingly, high levels of tumor necrosis were observed in tumors of patients having a mesenchymal subtype [4]. Furthermore, a recent study demonstrated that GBM cells surrounding necrotic zones and suffering from hypoxic conditions express high levels of the mesenchymal transcription factors C/EBP-β and C/EBP-δ and, in addition, the expression of these transcription factors was associated with a poor prognosis [7].

GBMs generally display rapid cell proliferation and inadequate vascularization leading frequently to tumor areas with insufficient oxygen supply [8]. This chronic exposure to extremely low levels of oxygen frequently produces necrotic zones surrounded by densely packed hypoxic tumor cells. These so-called pseudopalisading GBM cells were shown to express hypoxia-regulated genes that control crucial processes associated with tumor aggressiveness such as angiogenesis, extracellular matrix degradation, and invasive behavior [7,9]. Hypoxia is also a well-recognized component of the tumor microenvironment and has been linked to poor patient outcome and resistance to therapies in different cancer types [10–15].

The cellular responses to hypoxia are generally mediated by the hypoxia-inducible factor (HIF) family of transcription factors [16,17]. HIFs function as heterodimers composed of an oxygen-sensitive HIFα subunit and a constitutively expressed HIFβ subunit. Under normoxic

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conditions HIFα is subjected to proteasomal degradation as a result of ubiquitination by the von Hippel–Lindau (vHL) tumor-suppressor gene product. Under hypoxic conditions, however, the interaction between HIFα and vHL is abrogated and as a consequence of this the HIFα subunit is stabilized, thereby allowing dimerization with HIFβ and subsequent binding to hypoxia-responsive elements (HREs) in the promoters of hypoxia-regulated genes. In this way the transcription of hundreds of downstream genes are regulated that can modulate cell survival, motility, metabolism and angiogenesis in order to restore oxygen homeostasis [16,18,19]. Two HIFα subunits, HIF1α and HIF2α, are structurally similar in their DNA binding and dimerization domains, but show differences in their transactivation domain. HIF1α and HIF2α are known to have non-overlapping biological roles each having unique target genes and requiring different levels of oxygen for activation [20].

Hypoxia is a well-known inducer of the epithelial to mesenchymal transition (EMT) program in epithelial cancers like pancreatic ductal adenocarcinoma [21], hepatocellular carcinoma [22], ovarian carcinoma [23] and lung cancer [24]. EMT can contribute toward the invasion–metastasis cascade by inducing mesenchymal properties in tumor cells, including anoikis resistance and the ability to migrate and invade surrounding tissues [25]. Although the invasive phenotype of GBM is one of the major reasons for the poor prognosis associated with this disease, the involvement of hypoxia-induced mesenchymal transition has been hardly explored [26,27].

In the present study we examined whether hypoxia can induce a mesenchymal shift in GBM cells and explored the consequences for their invasive behavior and the underlying molecular mechanisms involved. We provide evidence for the concept that GBM cells undergo a mesenchymal transition in necrotic areas of the tumor thus facilitating the invasive behavior of the tumor.

Materials and methods

Cell lines and treatments

The human GBM cell lines U87 and SNB75 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and U251 was obtained from the CLS Cell Lines Service GmbH (Eppelheim, Germany). U87 was cultured on cell culture flasks pre-coated with 1% gelatine from porcine skin (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) in Dulbecco's modified Eagle's Medium high glucose (DMEM-Hg) (Gibco Life Technologies, Bleiswijk, The Netherlands) medium supplemented with 10% FCS and 1% penicillin(pen)/streptomycin(strep) (Gibco Life Technologies). SNB75 and U251 did not require gelatin coating of the flasks. Cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2. When indicated, cells were treated with the HIF1α and HIF2α inhibitor digoxin (Axon Medchem, Groningen, The Netherlands). The inhibitor was added at a concentration of 150 nM 2 hrs prior to exposing the cells to hypoxia.

For hypoxia treatment, cells were first maintained in the regular normoxic incubator for around 12 h until the cells attached to the flasks. Following this the flasks were transferred to the tri-gas incubator (Sanyo MCO 18M, from Sanyo E&E Europe BV, Etten-Leur, The Netherlands) filled with 1% O_2, 5% CO_2 and 94% N_2, at 98% humidity and 37 °C.

Western blotting

Preparation of protein lysates and Western blotting was carried out as described previously [28]. The membranes were probed with antibodies against HIF1α [1:1000, abcam (ab2185), Cambridge, UK], HIF2α [1:500, abcam (ab199)], SNAI1/SNAIL1 [1:500, Santa Cruz Biotechnology Inc (sc-10433), Santa Cruz, CA, USA], SLUG/SNAIL2 [1:500, Santa Cruz Biotechnology Inc (sc-166476)], ZEB1 [1:500, Santa Cruz Biotechnology Inc (sc-81428)], Twist antibody [1:1000 abcam, (ab50581)], Fibronectin [1:2500, BD Transduction Laboratories (610077), Franklin Lakes, NJ, USA], COL5A1 [1:2000, Santa Cruz Biotechnology, Inc (sc-20648)]. β-actin [1:10,000, MP Biomedicals (08691001), Duiven, The Netherlands] expression levels served as loading control. After incubation membranes were washed with TBST (20 mmol/l Tris–HCL (pH 8.0), 137 mmol/l NaCl and 0.1% Tween-20), and reprobed with appropriate HRP-conjugated secondary antibodies, anti-mouse immunoglobulin G (IgG), anti-rabbit IgG or anti-goat IgG) (Dako, Glostrup, Denmark) for 1 h at RT. Proteins were visualized by chemiluminescence using BM chemiluminescence detection kit (Roche Applied Science, Almere, The Netherlands).

Immunofluorescence microscopy

Cells cultured on poly L lysine (Sigma-Aldrich)-coated cover slips were fixed for 10 min using 4% formaldehyde. After 3 times washing with cold PBS, cells were permeabilized with 0.1% Triton (Sigma-Aldrich) in PBS, washed again with PBS followed by a blocking step for 1 h with PBS + 0.1% Tween-20 (Sigma-Aldrich), 2% BSA (PAA Laboratories GmbH, Colbe, Germany) and 1:50 dilution of normal goat serum (Dako). Subsequently, cells were incubated with the indicated primary antibodies at room temperature for 1.5 h. Primary antibodies used: purified mouse Fibronectin [1:100, BD Transduction Laboratories (610077)], COL5A1 [1:200, Santa Cruz Biotechnology (sc-20648)], HIF1α [1:1000, abcam (ab2185)], HIF2α [1:100, abcam (ab199)], ZEB1 [1:50, Santa Cruz Biotechnology Inc (sc-10572)]. After 4-times washing with PBS, slides were incubated for 1 h with the appropriate secondary antibodies: goat anti-Mouse Alexa 488 (1:200, Life Technologies), Donkey anti-goat Alexa 488 (1:200) or Goat anti-Rabbit IgG Antibody, Cy3 conjugate (1:400, Millipore (AP132 C)). Hoechst (Sigma H6024) staining was performed for 5 min followed by mounting the coverslips with Kaisers glycerin (Merck, Darmstadt, Germany). Cells were examined by fluorescent microscopy (Leica DM6000, Leica Microsystems GmbH, Mannheim, Germany) and images were captured using Leica DFC360 FX camera.

Transwell-invasion assay

The invasion potential was determined on Gelatin-coated Transwell inserts with 8 μm pore size (Becton Dickinson B.V., Breda, The Netherlands). For this, cells were trypsinized and resuspended in 0.1% FCS containing medium. 150 μl of a cell suspension containing 5×10^4 cells was added to the Transwell in triplicates per condition. 10% FCS or 0.1% FCS was added to the lower wells as chemoattractants. Cells that migrated/invaded and appeared on the bottom surface of the Transwell insert membrane were fixed with 75% methanol/25% acidic acid for 20 min and stained with 0.25% Coomassie blue in 45% methanol/10% acetic acid followed by washing with demi water. The membranes were subsequently cut out and mounted on microscopic slides for quantification. Representative pictures of the membranes with cells were acquired at 5× magnification and the total number of cells on fifty individual fields per membrane was counted; average numbers and standard deviation of invading cells for every condition were calculated.

Wound healing assay

The migratory capacity of cells was determined by wound healing assays. Briefly, 2×10^5 cells were seeded on poly-L-Lysine (Sigma-Aldrich) coated 6 well plates in culture medium; upon confluency a scratch was made using a P10 pipette tip. The rate of wound closure was monitored at different time points under a microscope.

Short interfering RNA silencing

Validated Stealth RNAi (OriGene SR304746, Rockville, MD, USA) specific for ZEB1 was transfected into U87 cells by using Lipofectamine RNAiMAX (Invitrogen, Leek, The Netherlands) according to the manufacturer's protocol. Trilencer-27 Universal scrambled negative control siRNA (OriGene SR30004) was used as negative control. For shRNA silencing, a lentiviral vector expressing a short hairpin against HIF1α was made by cloning the hairpin sequence from pSuper-puro-HIF1a1470 (which was a kind gift from Daniel Chung, Massachusetts General Hospital, Boston, Massachusetts) into the pLVUT vector [29]. A short hairpin sequence against HIF2a was constructed by cloning the hairpin sequence from pRetro-Super-HIF2a (obtained from Addgene, Cambridge, MA, USA, number 22100) into the pLVUT vector. A control vector was made by cloning a hairpin against firefly luciferase into the pLVUT vector. Viral particles were generated and lentiviral transductions were performed to generate stable knockdowns as previously described [29]. In summary, lentiviral particles were harvested in DMEM (hg) medium and stored at –80 °C until further use. Cultured U87 cells were transduced in 3 consecutive rounds within 8–12 h intervals between each round with lentiviral supernatant supplemented with polybrene (0.004 mg/ml).

Immunohistochemistry

Formalin fixed paraffin-embedded 3 μm thick consecutive tissue sections were mounted on microscope slides and dried overnight at 55 °C. Tissue sections were deparaffinized in xylol and rehydrated in graded series of ethanol, and stained with hematoxylin and eosin (HE). Antigen retrieval was performed using microwave pretreatment in pH 6.0 citrate buffer. Sections were treated with 0.3% H_2O_2 for 30 min and blocked for 1 h with 2% BSA to reduce non-specific primary antibody binding. Incubation with the following antibodies was performed overnight at 4 °C: rabbit anti-ZEB1 [1:150, Sigma-Aldrich], rabbit anti-Glut1 [1:100, Abcam], goat anti-YKL40/GP39 [1:200, Santa Cruz Biotechnology Inc].

As negative controls, primary antibodies were omitted. After incubation with primary antibodies suitable secondary antibodies conjugated to peroxidase (Dako) and appropriate tertiary antibodies conjugated to peroxidase (Dako) were used. Staining was visualized by 3,3′-diaminobenzidine and sections were counterstained with hematoxylin and mounted. Images of relevant sections were acquired using a C9600 NanoZoomer (Hamamatsu Photonics KK, Hamamatsu City, Japan).

Statistical analysis

In-vitro data of three independent experiments were represented as the mean ± standard error of the mean (SEM) in the form of graphs using the GraphPad Prism version 5.01 (GraphPad for Science, San Diego, CA). Statistical significance was calculated by two way unpaired Student's t-test unless otherwise mentioned in the figure legends. p values < 0.05 were assumed as statistically significant for all the tests.

Results

Hypoxia induces a phenotype shift and increases migration/invasion in GBM cells

The exposure of U87, SNB75 and U251 cells to hypoxia (1% O_2) for 72 h resulted in a marked difference in their morphology compared to normoxia (20% O_2) cultured cells particularly in U87 and SNB75. Under hypoxic conditions the cells had a more elongated morphology and were more loosely clustered than normoxia cultured cells (Fig. 1a). The migration/invasion potential of U87 cells was tested using gelatin-coated transwell inserts. Hypoxia exposed cells demonstrated almost double the amount of migratory/invasive cells in comparison to cells cultured under normoxic conditions (Fig. 1b, c).

Hypoxia promotes mesenchymal transdifferentiation that is associated with accumulation of nuclear HIF1α, HIF2α and ZEB1

We next explored if the hypoxia-dependent change in morphology could be the result of mesenchymal transition in GBM cells. Therefore the expression of several mesenchymal markers was examined by immunofluorescence microscopy in U87, SNB75 and U251 cells under hypoxic and normoxic conditions. Previously we found U87 and U251 to represent predominantly the classical (CL)/PN subtype [28], and SNB75 cells also appeared to be mostly non-mesenchymal lacking Fibronectin and COL5A1 expression (Fig. 2b). A strong induction of Fibronectin and COL5A1 expression was seen under hypoxic conditions in U87 and SNB75 (Fig. 2a, b), whereas the basal expression levels of YKL40 and Vimentin were not altered (not shown). In contrast, in U251 cells no such inductions were observed (Fig. 2c) and rather a reduction in migration potential under

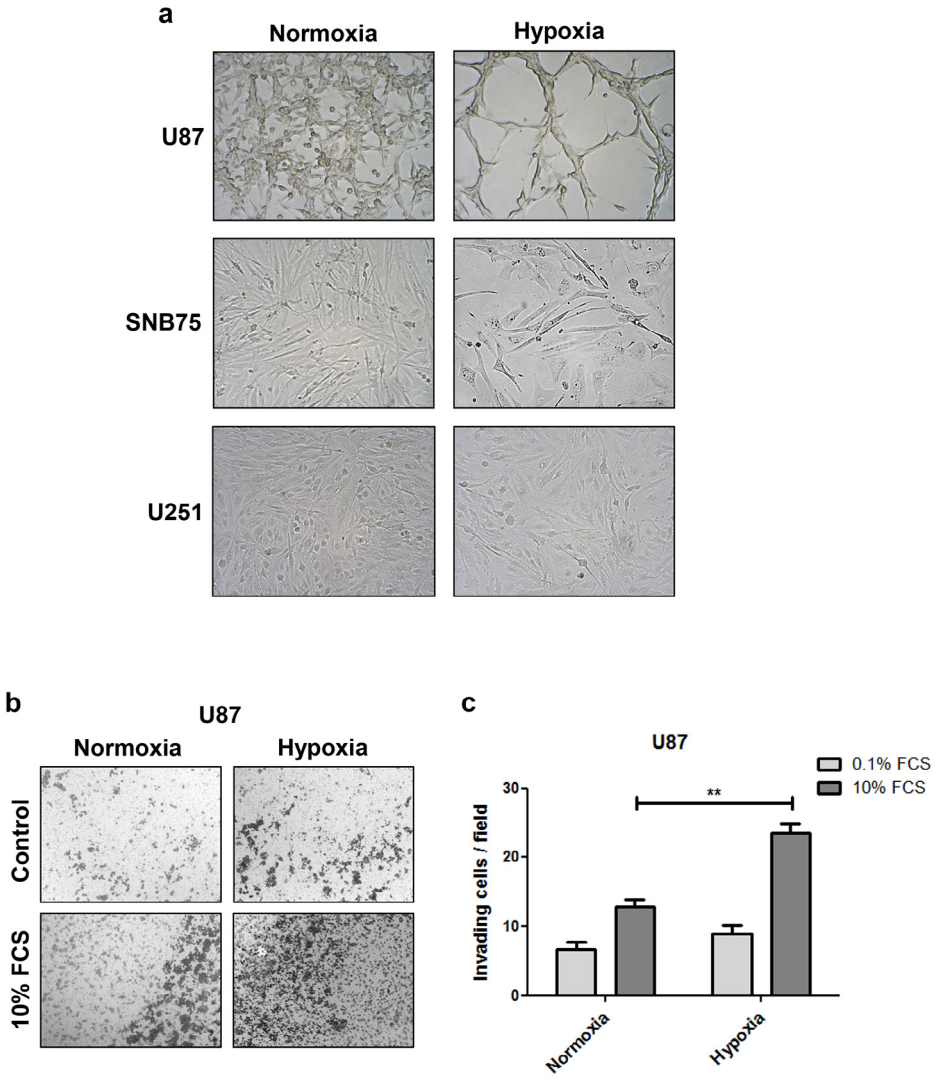

Fig. 1. Hypoxia induces a phenotypic shift and enhances the invasive phenotype in GBM cells. (a) Representative Phase contrast microscopic pictures (×10) of U87 and SNB75 cells undergoing phenotypic shift characterized by a more elongated and stretched morphology under hypoxia in comparison to the cells in normoxia; U251 cells appear less affected by hypoxia. (b) A representative transwell assay showing Coomassie blue stained U87 cells on the insert membranes, demonstrating enhanced invasive capacity under hypoxic conditions in comparison to cells under normoxia. 0.1% FCS containing media was used as the control and 10% FCS served as the chemo-attractant. (c) Quantified data of the invasion assay are depicted as mean of three independent experiments measured in triplicate ± SEM (**p < 0.01).

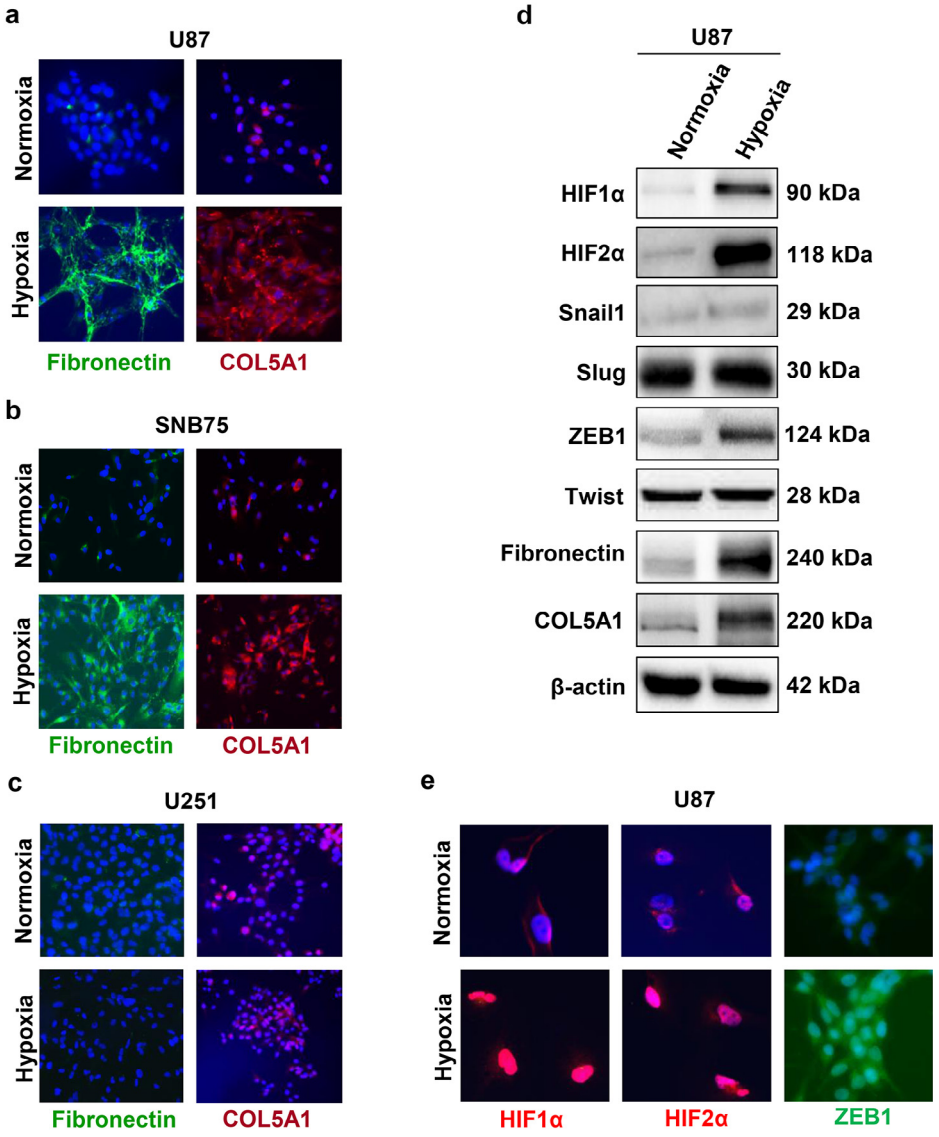

Fig. 2. Hypoxia induces a mesenchymal shift in GBM. (a–c) Immunofluorescence analysis for mesenchymal markers (Fibronectin and COL5A1) in U87, SNB75 and U251 cells exposed to hypoxia in comparison to these cells grown under normoxic conditions; images obtained at ×20 magnification. (d) Western blots showing the expression levels of multiple markers associated with a mesenchymal phenotype in U87 cells under hypoxia conditions. (e) Immunofluorescence analysis revealing nuclear localization of HIF1α, HIF2α and ZEB1 in U87 cells exposed to hypoxia; images obtained at ×40 magnification.

hypoxic conditions was observed in wound-healing assays (Supplementary Fig. S1a, b).

To explore the underlying mechanism of the hypoxia-induced mesenchymal shift we employed U87 cells and performed western blot analyses to evaluate the expression levels of HIF1α and HIF2α along with EMT inducing transcription factors such as Snail1, Snail2/Slug, ZEB1 and TWIST, and Fibronectin and COL5A1. The expression of both HIF1α and HIF2α was strongly induced together with the mesenchymal markers Fibronectin and COL5A1, and out of the transcription factors tested we could detect only ZEB1 to be significantly upregulated under hypoxia (Fig. 2d). Of note, we detected the 124 kDa form of ZEB1 and not the larger ~200 kDa form, which are both known to be specific for ZEB1 [30]. Further, immunofluorescence analysis revealed nuclear localization of HIF1α, HIF2α and ZEB1 in U87 cells under hypoxic conditions, while under normoxia little or no nuclear expression of these transcription factors was detected (Fig. 2e). Of note U251 cells that did not demonstrate a hypoxia-induced gain in mesenchymal marker expression showed nuclear translocation of HIF1α, but not of ZEB1 (Supplementary Fig. S1c, d). Time course experiments showed accumulation of HIF1α, HIF2α and ZEB1 after 6 and 12 h hypoxia exposure and mesenchymal differentiation became evident after 48 h exposure as indicated by the appearance of Fibronectin expression (Supplementary Fig. S2). Thus, these results suggested the involvement of HIF1α, HIF2α and ZEB1 in inducing a mesenchymal shift.

Digoxin effectively inhibits the hypoxia-induced mesenchymal shift and increase in invasion

To investigate further the role of HIFs in the hypoxia-induced mesenchymal shift, we employed a cardiac glycoside, digoxin, a well-known inhibitor of HIF1α and HIF2α. Digoxin is known to have modest effects on global protein synthesis but is a very potent inhibitor of HIF1α mRNA translation [31]. Different concentrations of digoxin were tested in hypoxia-exposed U87 cells to examine which dose was effective in blocking the phenotypic shift. A concentration of 150 nM appeared effective and was also able to inhibit the accumulation of HIF1α and Fibronectin at the protein level

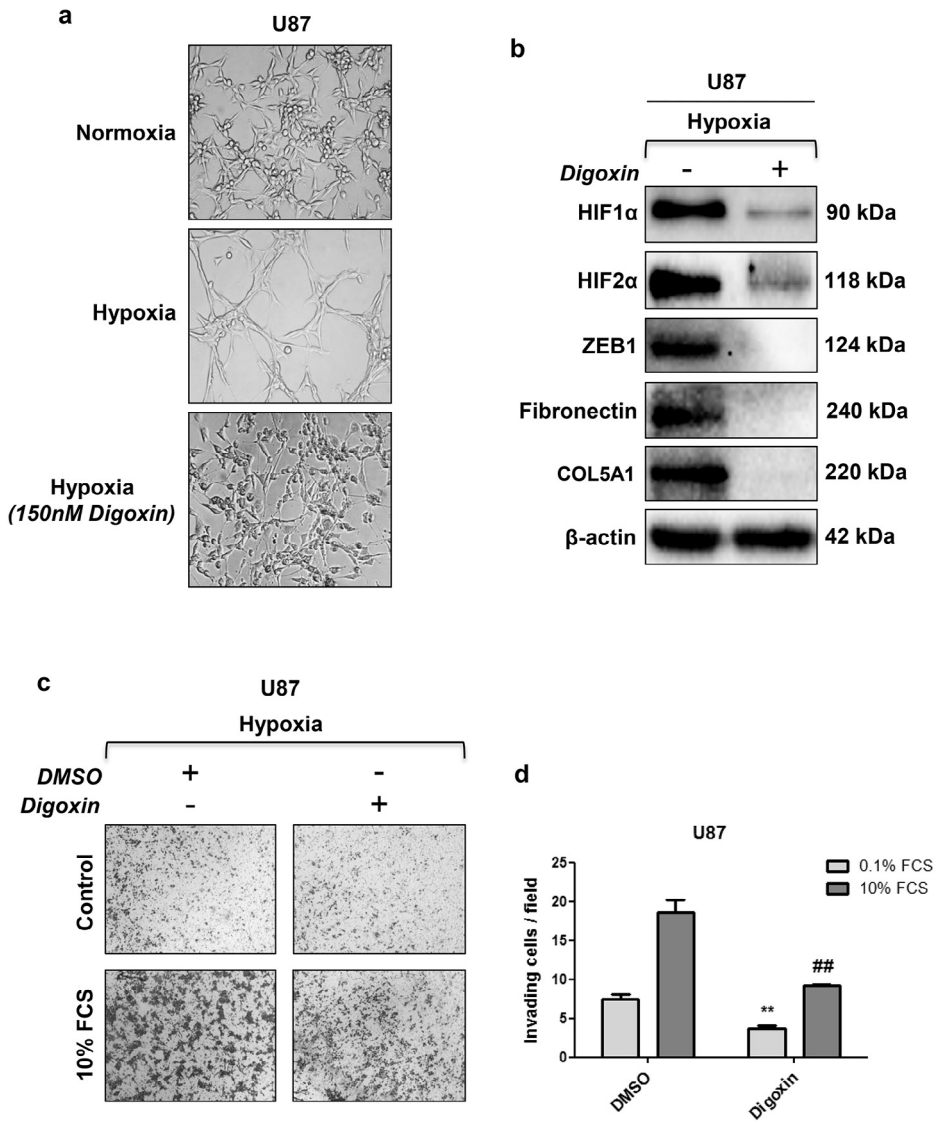

Fig. 3. Digoxin prevents the hypoxia-induced mesenchymal shift and elevated invasion. (a) Representative phase contrast microscopic pictures (×10 magnification) showing the effect of hypoxia on the morphology of U87 cells in the presence and absence of digoxin. (b) Western blots showing the effect of digoxin on the expression of the indicated proteins in hypoxia exposed U87 cells. (c) A representative transwell assay showing Coomassie blue stained U87 cells on the membrane inserts and quantified results are depicted in (d). The bars represent the mean of in general three independent experiments measured in triplicate ± SEM (**$p < 0.01$, DMSO vs digoxin for 0.1% FCS and ##$p < 0.01$ DMSO vs digoxin for 10% FCS).

(Supplementary Fig. S3a, b). Higher concentrations of digoxin appeared toxic to the cells. Digoxin added 2 h prior to placing the cells under hypoxia effectively prevented the phenotypic shift and western blot analysis further revealed that digoxin also prevented largely the induction of HIF1α and HIF2α together with that of ZEB1, Fibronectin and COL5A1 (Fig. 3a, b). Next we examined the effect of digoxin on the hypoxia-dependent increase of migration/invasion in U87 cells; a significant reduction (~2 fold) in the invasive potential of these cells was seen (Fig. 3c, d), indicating essential roles of HIF1α and/or HIF2α in mesenchymal differentiation and enhanced invasive potential.

HIF1α is instrumental for the induction of ZEB1, a mesenchymal shift and increased migration/invasion under hypoxia

We proceeded by examining which of the two HIFs are instrumental for inducing a mesenchymal shift. U87 cells were generated in which either HIF1α or HIF2α expression was stably silenced using selective shRNAs and control scrambled shRNA encoded by lentiviral vectors. Effective knockdown was confirmed at the protein level by western blotting and we found that the silencing of HIF1α and not HIF2α effectively prevented the induction of ZEB1 and Fibronectin expression under hypoxia (Fig. 4a). In line with this, control and HIF2α knockdown cells showed the characteristic shift in morphology upon hypoxia, whereas HIF1α knockdown cells appeared similar to normoxia cultured U87 cells (Fig. 4b). The HIF1α knockdown cells also showed a clear decline in the invasive potential in comparison to the HIF2α knockdown and control cells under hypoxic conditions (Fig. 4c, d).

ZEB1 mediates the hypoxia-induced mesenchymal shift and invasive phenotype

To further examine the role of ZEB1 we silenced the expression of ZEB1 with 2 different specific siRNAs in comparison to scrambled siRNAs. Exposure of siZEB1-I and -II-transfected U87 cells to hypoxia prevented the phenotypic shift while the control cells (mock and the scrambled siRNA treated) showed a visible

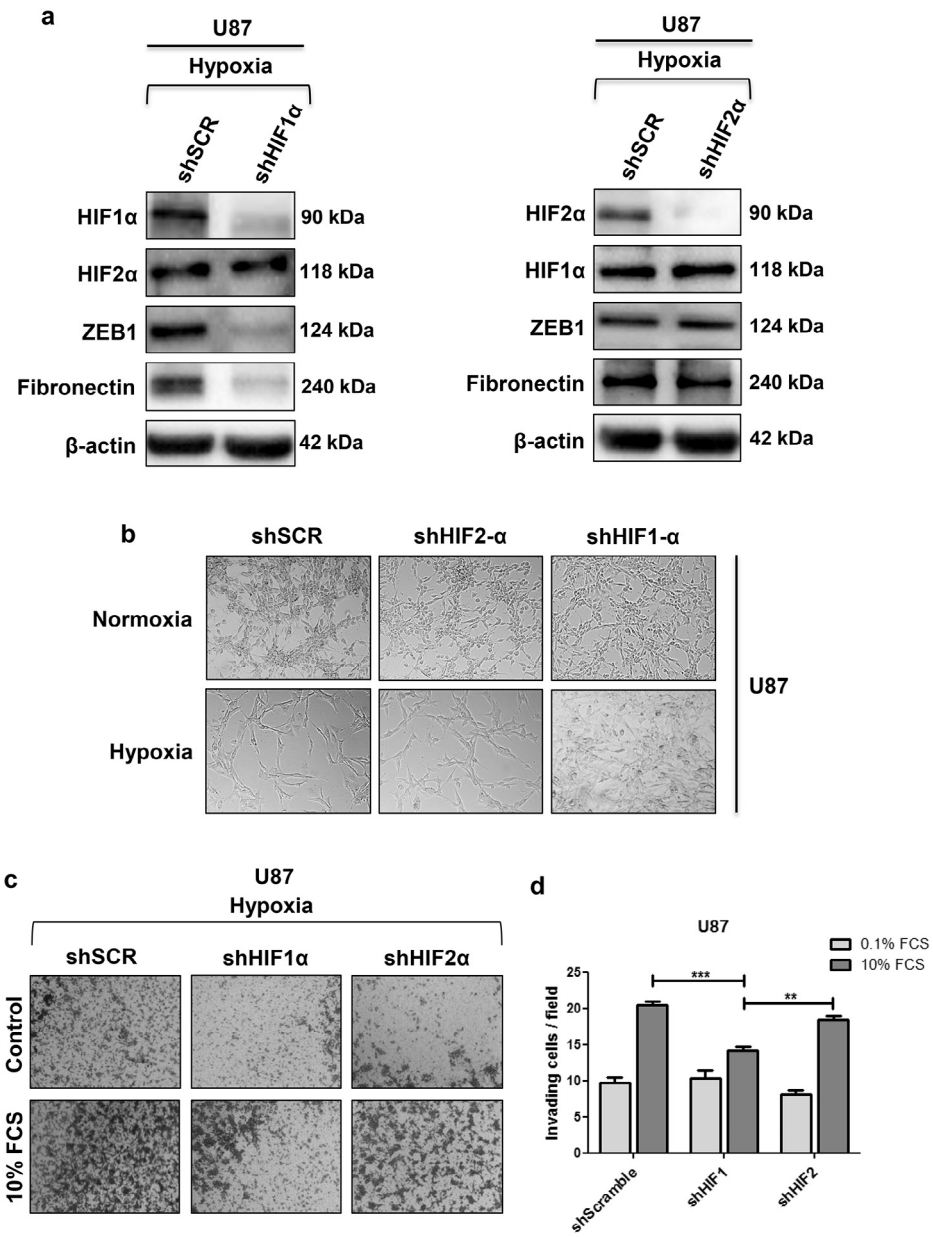

Fig. 4. HIF1α knockdown prevents hypoxia-induced mesenchymal transition. (a) Western blot analyses showing effective downregulation of HIF1α and HIF2α in U87 cells following ShRNA-mediated gene silencing and exposure to hypoxia when compared to control scramble shRNA (shSCR). The effects on ZEB1 and Fibronectin expression were also examined. (b) Representative phase contrast microscopic pictures (×10 magnification) of U87 cells transfected with shHIF2α or shSCR undergoing a phenotypic shift under hypoxia, whereas U87-ShHIF1α cells largely retained their original morphology under hypoxic conditions. (c) Representative pictures of transwell assays showing Coomassie blue stained U87 (shSCR, shHIF1α, shHIF2α) cells on the membrane inserts. A decreased invasive potential of HIF1α knockdown cells under hypoxia was seen. 0.1% FCS containing media was used as the control and 10% FCS served as the chemo-attractant. Quantified data for invading cells/field are shown in (d) as mean ± SEM of 3 independent experiments (**$p < 0.01$ and ***$p < 0.001$).

morphological shift under hypoxia (Fig. 5a). Western blotting confirmed inhibition of ZEB1 induction by hypoxia in the specific ZEB1 siRNA transfected U87 cells together with an absence of Fibronectin and COL5A1 accumulation (Fig. 5b). In ZEB1 knockdown cells HIF1α induction remained as strong as in control cells indicating that HIF1α acts upstream of ZEB1 in triggering a mesenchymal shift under hypoxia (Fig. 5b). Significant reduction in the invasive potential was also observed in U87 cells under hypoxia following the silencing of ZEB1 (Fig. 5c, d). This indicates that ZEB1 is a crucial mediator of the hypoxia-induced HIF1α dependent mesenchymal shift and the invasive phenotype of these GBM cells.

Overlapping GLUT1, ZEB1 and YKL40 expression in patient material

Finally, in order to examine whether hypoxia-induced mesenchymal transition may also be relevant in patient tumors, we selected GBM patient material showing multiple pseudopalisading necrotic regions as was determined by H&E staining (Fig. 6a). Subsequently, we performed immunohistochemical staining on serial sections made from this material for GLUT1, a hypoxia marker, ZEB1 and the mesenchymal marker YKL40. Interestingly, we noticed an overlapping expression of these markers in the hypercellular zones/pseudopalisades surrounding the necrotic foci (Fig. 6b). Taken

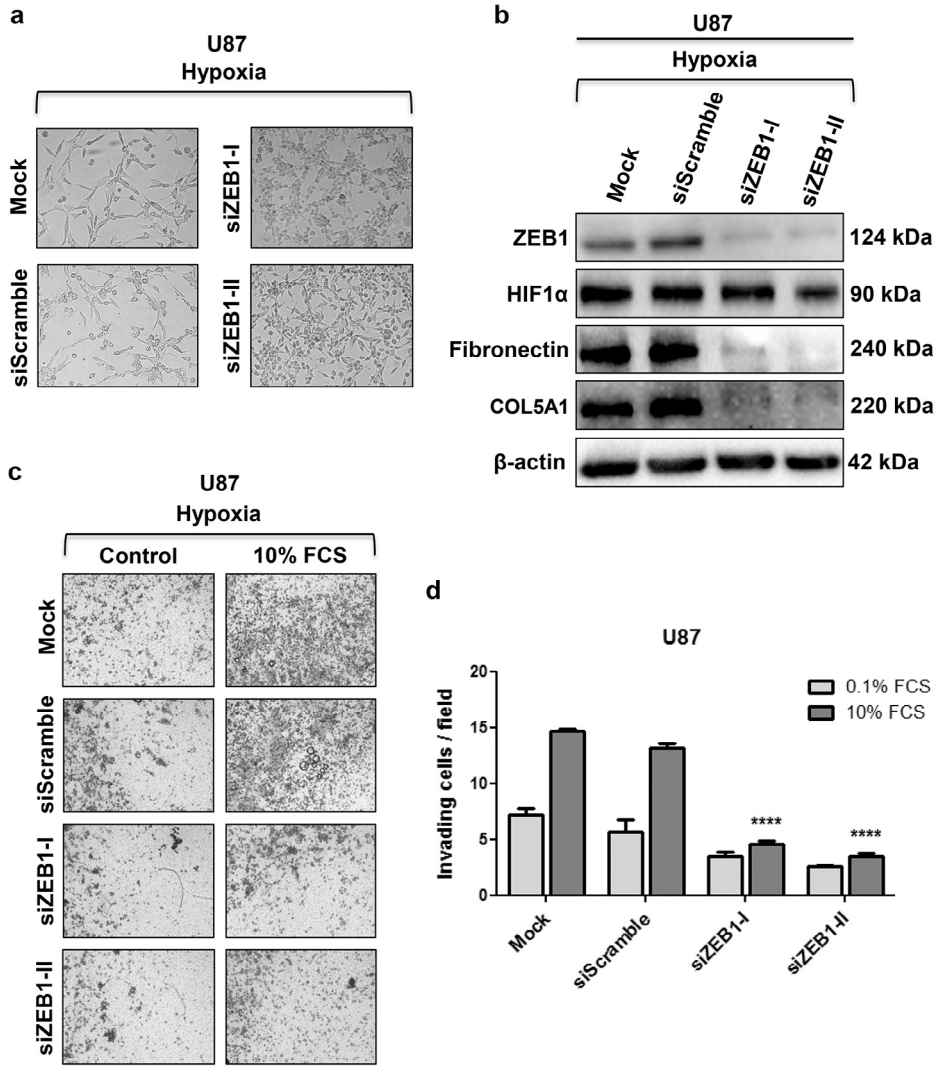

Fig. 5. ZEB1 silencing effectively prevents the hypoxia-induced mesenchymal shift and invasion. (a) siRNA silencing of ZEB1 effectively prevented the hypoxia-induced phenotypic shift in U87 cells in comparison to the Mock and Si-negative control treated cells; representative phase contrast microscopic pictures (×10) are shown. (b) Western blot analysis showing downregulation of ZEB1 following siRNA administration; levels of HIF1α, Fibronectin and COL5A1 are also shown. (c) A representative Transwell assay showing Coomassie blue stained U87 cells on the membrane inserts, demonstrating decreased invasive potential under hypoxia following silencing of ZEB1 expression in comparison to the Mock and negative siRNA treated cells. Quantification of invading cells is shown in (d) where bars indicate the average invading cells/field in 3 independent experiments ± SEM. (****$p < 0.0001$ between ZEB1 targeted siRNA-treated cells vs scrambled siRNA).

together this provides further evidence for a link between hypoxia and ZEB1-mediated mesenchymal transition in GBM.

Discussion

Hypoxic regions are frequently found in GBM and the presence of extensive hypoxic areas has been associated with worse prognosis in GBM patients, which has been linked to hypoxic cancer cells displaying a more malignant phenotype and being more resistant to chemotherapy and radiation [32–34]. The HIF transcription factors are instrumental for orchestrating adaptive responses to cope with oxygen shortage, and particularly HIF1α is key in inducing expression of glycolytic enzymes and several angiogenic growth factors [12,17,35]. HIF1α was found to be upregulated in many of the malignant tumors primarily by hypoxia-mediated protein stabilization [12].

In GBM, HIF1α seems to be primarily localized to the pseudopalisading cells around necrotic cores and to tumor cells at the invasive edge of the tumor that infiltrate the normal brain tissue [36]. Extensive necrosis and elevated levels of HIF-regulated genes are features that were more frequently found in mesenchymal GBM when compared to proneural GBM [4,6,37]. Despite the association between hypoxia and mesenchymal GBM, the potential molecular mediators that induce a mesenchymal shift under hypoxic condition remain a poorly studied area.

In the present study we showed that hypoxia is a strong inducer of a mesenchymal shift in GBM that was associated with morphological changes, upregulation of known mesenchymal markers like Fibronectin and COL5A1 and elevated invasive potential in vitro. We independently tested the role of the two HIFα proteins – HIF1α and HIF2α, as we observed the up-regulation and nuclear translocation of both of these HIF proteins under hypoxic conditions. HIF1α is the most well studied member of the HIFα family due to its universal pattern of expression, unlike HIF2α that shows a more restricted expression pattern. Notably, HIF2α has been reported to play a crucial role in regulating stemness in GBM [38–40]. We found that hypoxia-induced HIF1α, and not HIF2α, is a key mediator for mesenchymal transition. The EMT transcription factor ZEB1 known to regulate EMT in epithelial cancers [41,42] appeared instrumental in this transition since siRNA-dependent silencing of ZEB1

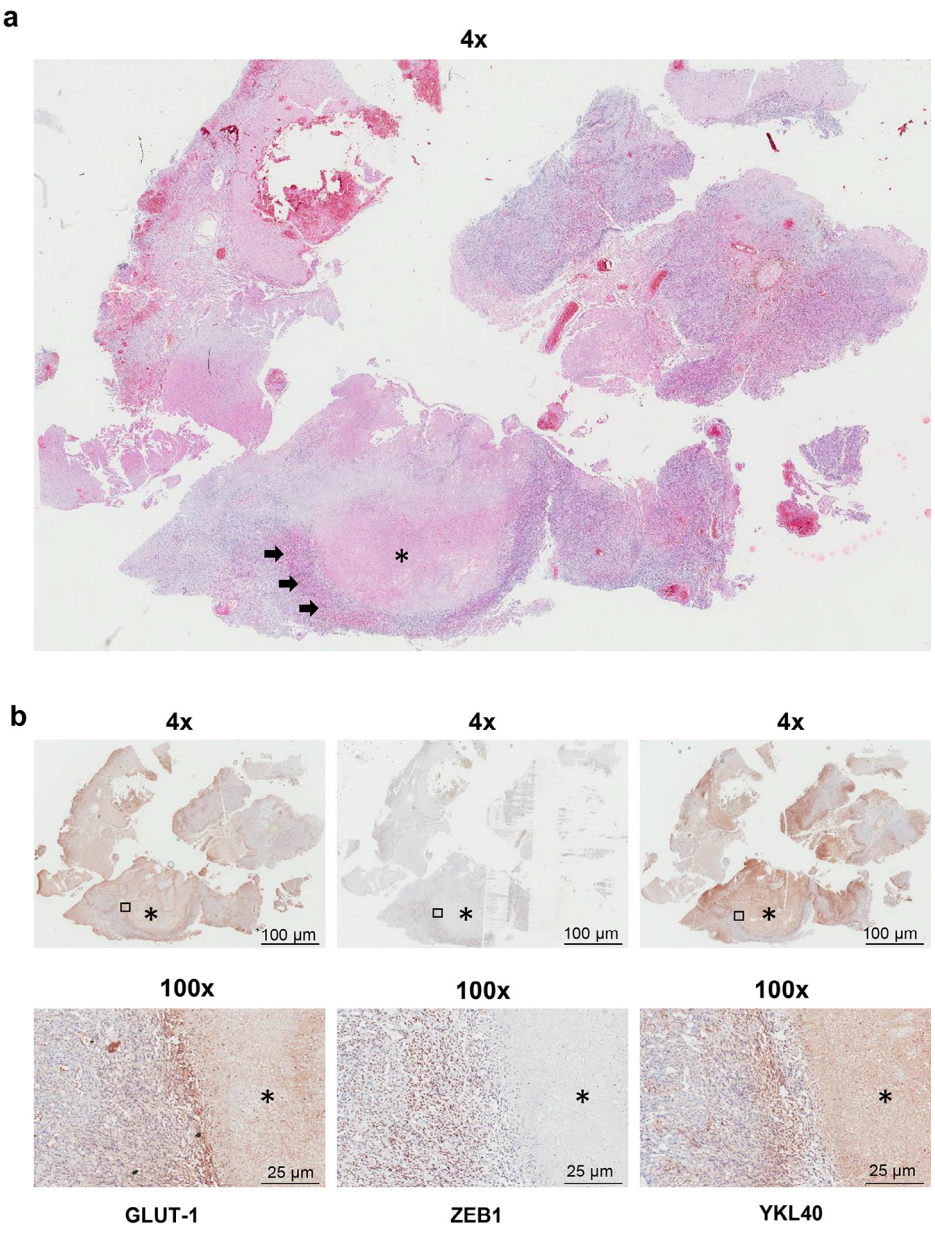

Fig. 6. Local/regional mesenchymal transition detected in hypoxic zones in GBM patient material. (a) Photomicrograph of hematoxylin–eosin (H&E) staining [original magnification ×4] in GBM patient material showing pseudopalisading necrosis characterized by a garland-like arrangement of hypercellular tumor nuclei (arrows) lining up around irregular foci of tumor necrosis. (b) Immunohistochemical staining for GLUT1, ZEB1 and YKL40 in consecutive sections detects overlapping expression patterns of these markers in pseudopalisading cells [original magnification ×100]. The areas indicated with boxes are enlarged. *Indicates necrotic region.

prevented the hypoxia induced mesenchymal shift and enhanced invasive capacity. Our finding of overlapping ZEB1, GLUT1 and YKL40 expression patterns surrounding necrotic areas in GBM patient material provides evidence for the occurrence of local hypoxia-induced ZEB1-mediated mesenchymal transition in these tumors. Interestingly, ZEB1 has been recently associated with invasive behavior, temozolomide resistance and stemness in GBM [43]. Moreover, we previously identified a critical role for ZEB1 in mediating a TGF-β-induced mesenchymal shift in GBM cells [28]. Furthermore, the TNFα/NF-κB and WNT/β-catenin pathways were also reported to be able to mediate a mesenchymal shift in GBM [44,45]. Of note, hypoxia did not trigger a mesenchymal shift in U251 cells, whereas previously we found that these cells underwent such transdifferentiation upon TGF-β exposure [28]. Apparently, some GBM cells are refractory to one mesenchymal-inducing stimulus while being sensitive to others providing multiple ways for GBM cell to acquire the aggressive mesenchymal status. The mesenchymal phenotype in GBM, in addition to being regulated by the transcription factors C/EBP-β, STAT3 and TAZ [6,46], is thus controlled by various external stimuli. How and whether these mechanisms are further interlinked remains to be investigated.

In summary, as indicated in (Fig. 7) hypoxia induces a mesenchymal shift in GBM that is mediated by the HIF1α–ZEB1 axis leading to an elevated invasive potential. Our results further indicate the crucial role of micro-environmental factors like hypoxia in defining GBM sub-types and thus the boundaries between these molecular subtypes appear less strict than initially believed. Hence, therapeutic targeting of HIF1α or its downstream target ZEB1 might provide a possible strategy for improving the prognoses for GBM patients.

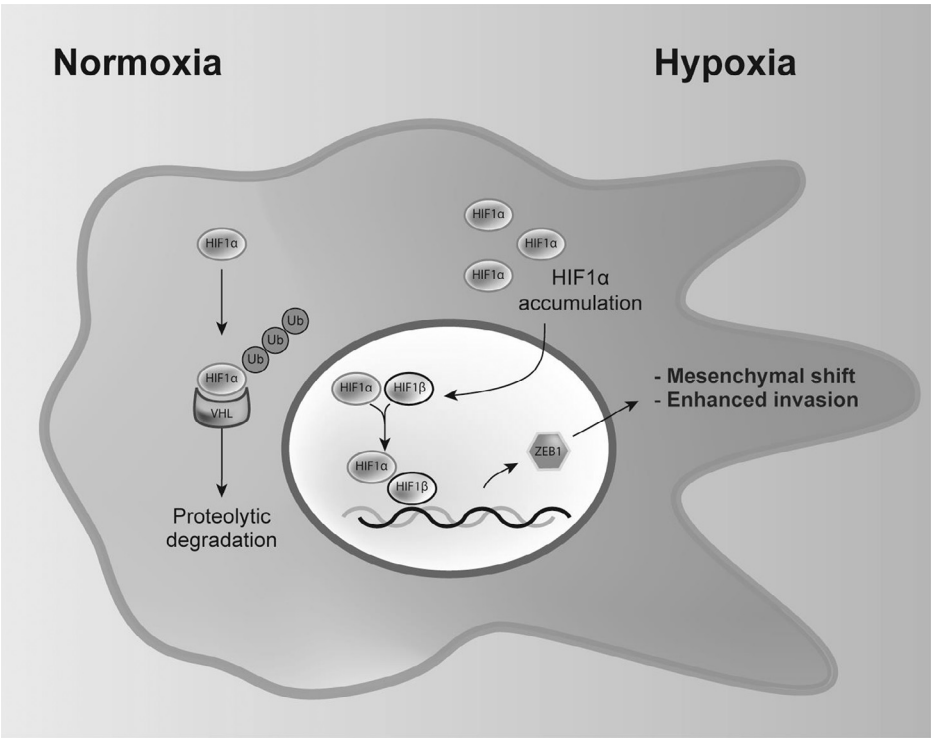

Fig. 7. Model illustrating hypoxia induced mesenchymal transition in GBM that is mediated by HIF1α and ZEB1. Mesenchymal transition leads to heterogeneity in GBM subtype and leads to a gain of an invasive phenotype.

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Conflict of interest

The authors have no conflict of interests.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.01.010.

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